TAXODIUM (CUPRESSACEAE): ONE, TWO OR THREE SPECIES? EVIDENCE FROM DNA SEQUENCES AND TERPENOIDS

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ABSTRACT

DNA sequences (3547 bp) of nrDNA and cp regions (petN-psbM, trnS-trnG, ycf-psbA) were utilized to examine variation in *Taxodium distichum*, *T. d.* var. *imbricarium* and *T. mucronatum* using *Glyptostrobus pensilis* as an out-group. In contrast to considerable geographical variation found in the terpenoids (Adams et al. 2012), no informative mutations were found in these DNA sequences. The 16 SNPs found in nrDNA did not support three species of *Taxodium*. No variation was found in the cpDNA regions. DNA sequencing and terpenoids support the recognition of *Taxodium* as a monotypic genus with three varieties, var. *distichum* (L.) Rich. var. *imbricarium* (Nutt.) Croom and var. *mexicanum* (Carr.) Gord. *Phytologia 94(2): 159-168 (August 1, 2012).*

KEY WORDS: *Taxodium distichum*, *T. d.* var. *imbricarium* (*T. ascendens*), *T. d.* var. *mexicanum* (*T. mucronatum)*, nrDNA, cpDNA.

Taxodium Rich. is a small genus with one to three species. Britton (1926), Dallimore and Jackson (1966) and Rehder (1940) recognized three species: bald cypress, *T. distichum* (L.) Rich, pond cypress, *T. ascendens* Brongn. and Montezuma or Mexican bald cypress, *T. mucronatum* Ten. Watson (1985) treated *T. ascendens* as *T. d.* var. *imbricarium* (Nutt.) Croom. Based on morphology, Farjon (2005) and Eckenwalder (2009) recognized *T. distichum*, *T. d.* var. *imbricarium* and *T. mucronatum*. However, Denny (2007) treated the genus as monotypic with one species, *T. distichum* and three varieties: var. *imbricarium* and var. *mexicanum* (Carr.) Gord. (= *T. mucronatum*). Denny (2007), and Denny and Arnold (2007), give a lucid discussion of the historical nomenclature of the genus.

Recently, we reported on the leaf essential oils of *Taxodium distichum*, *T. d.* var. *imbricarium* (Adams et al. 2012). The geographical trends in the oils are shown in Fig. 1. *T. mucronatum* in

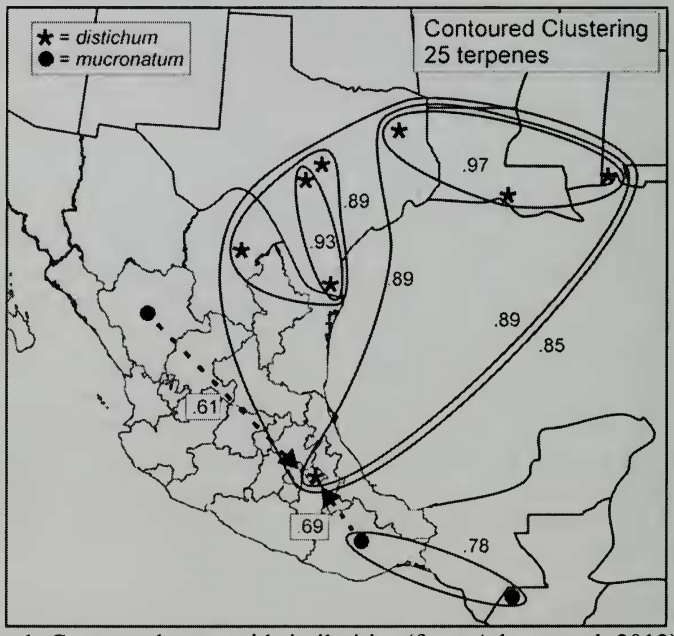

Figure 1. Contoured terpenoid similarities (from Adams et al. 2012). The number next to a contour line is the clustering similarity.

Mexico appears to be of two oil types: Durango and Oaxaca-Guatemala. The oils of *T. distichum* are in two regional groups: South Central USA and Texas Hill Country - Rio Grande Valley. The oil of the putative *T. mucronatum* from Bolleros, MX is more similar to *T. distichum* than to any *T. mucronatum* in that study (Adams et al. 2012). Putative *T. d.* var. *imbricarium* from Fowl River, AL was found to have oil like *T. d.* var. *distichum* not like *T. d.* var. *imbricarium* from Tampa, FL. Both *T. d.* var. *distichum* and var. *imbricarium* were found to have chemical races: high/low α-pinene and low/high limonene/β-phellandrene.

The purpose of this paper to is to report on variation in DNA sequences (3547 bp) of nrDNA and three cp regions (petN-psbM, trnS-trnG, ycf-psbA) and relate this DNA data to the taxonomy of *Taxodium*.

MATERIALS AND METHODS

Plant material: Seeds were collected by Denny (2007) in late summer and fall, 2003 and germinated and grown in containers in spring, 2004. Subsequently, seedlings were transplanted to the Texas A & M University Horticulture Farm, College Station, Texas (30°37'38.222" N, 96°22'18.505" W).

T. mucronatum: Rio Nazas, Durango, MX, MX2M, 25° 18' 36" N, 104° 38' 24" W, *Adams 12833-12837*; Rio Sabinas, Coah., MX, *Adams 13039-13043*, Bolleros, DF, MX, MX3M, 19° 30' 0" N, 98° 54' 36" W, *Adams 12838-12842;* Progreso, TX, MX5M, 26° 4' 12" N, 97° 54' 36" W, *Adams 12843-12847.*

T. distichum: Guadalupe River, TX, TX2D, 30° 4' 12" N, 99° 17' 24" W, *Adams 12848-12852*; Sabinal River, TX, TX5D, 29° 9' 36" N, 99° 28' 12" W, *Adams 12853-12857;* Lake Cherokee, TX, EP1D, 32° 20' 24" N, 94° 42' 0" W, *Adams 12858-12862;* Bayou Teche, LA, EP3D, 29° 5' 24" N, 91° 12' 6" W, *Adams 12863-12867*; Mobile Bay, AL, EP4D, 30° 36' 0" N, 87° 54' 36" W, *Adams 12868-12872.*

T. distichum var. *imbricarium*: Fowl River, AL, EP5I, 30° 27' 0" N, 99° 06' 36" W, *Adams 12873-12877.*

In addition, samples were collected from:

T. distichum: Hillsborough River, Hillsborough Co., FL, 28° 09' 05.15" N, 82° 13' 37.24" W, *Adams 12828*, Hillsborough River,

Hillsborough Co., FL, 28° 01.164' N, 82° 27.881' W, *Adams 12829-12830,* Hillsborough River, Hillsborough Co., FL, 27° 59.796' N, 82° 28.010' W, *Adams 12831-12832*;

T. d. var. *imbricarium*: edge of swamp, Hillsborough Co., FL, 28° 11' 39.80" N, 82° 30' 54.09" W, *Adams 12823-12827.*

Seeds were removed from a herbarium specimen (TEX) of *M Veliz 17213*, 31 Aug 2006, Chimaltenango, Guatemala, Lab Acc. *12591* for DNA extraction.

Specimens of *Glyptostrobus pensilis* K. Koch were obtained from the Stephen F. Austin State University, Mast Arboretum for use as an out-group to *Taxodium* (Kusumi et al. 2000). Voucher specimens are deposited in the Herbarium, Texas A & M University (for plot materials) and Baylor University (for Adams field collected materials).

DNA Analysis - One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted using the Qiagen DNeasy mini kit (Qiagen Inc., Valencia CA). PCR amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN-psbM, trnS-trnG) or K (nrDNA, ycf-psbA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary enhancers with 1.5 - 3.5 mM $MgCl_2$ according to the buffer used) 1.8 µM each primer. See Adams and Schwarzbach (2011) for the ITS, petN-psbM, and trnS-trnG primers utilized. Primers used for ycf exon 3 - spacer - psbA: ycf1843F GCT CCA AGC AAT TAT ATC GAA GCA CA; psbA2516R ATG ATC TTT ACT TCT GGT TCC GGT GA. The PCR reaction was subjected to purification by agarose gel electrophoresis (1.5% agarose, 70 v, 55 min.). In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit. The gel purified DNA band with the appropriate primer was sent to McLab Inc. (South San Francisco, CA) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.). Alignments and NJ trees were made using MAFFT (http://align.bmr.kyushu-u.ac.jp/mafft/). Minimum spanning networks were constructed from SNPs data using PCODNA software (Adams, 2009). Associational measures were computed using absolute compound value differences (Manhattan metric), divided by the

maximum observed value for that compound over all taxa (= Gower metric, Gower, 1971; Adams, 1975). Principal coordinate analysis was performed by factoring the associational matrix based on the formulation of Gower (1966) and Veldman (1967).

RESULTS AND DISCUSSION

Sequencing nrDNA yielded 65 SNPs (4 single events) with 61 potentially informative SNPs. 55 of these SNPs differentiated *Glyptostrobus pensilis* from *Taxodium* (Fig. 2). For the remaining 15

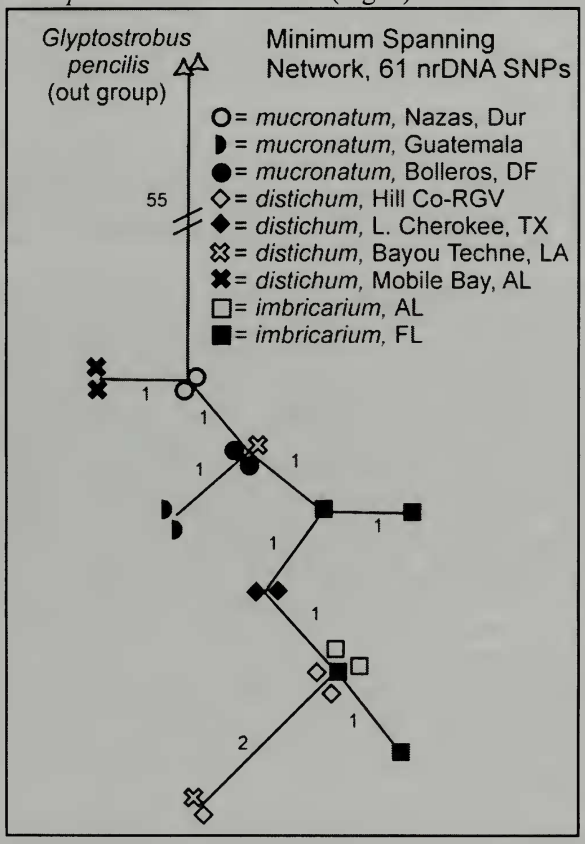

Figure 2. Minimum spanning network based on 61 SNPs from nrDNA. The numbers next to the links are the number of SNPs.

SNPs, the pattern appears to be mostly random (Fig. 2). Although the *T. mucronatum* from Durango, Bolleros and Guatemala differ by only 1 SNP, they also differ by only one SNP from *T. distichum*, Mobile Bay, AL and *T. d.* var. *imbricarium*, FL. *Taxodium d.* var. *imbricarium*, FL is interspersed with *T. distichum*, Hill Country- RGV and Lake Cherokee, TX (Fig. 2). There is no apparent grouping of the three taxa.

Sequencing of petN-psbM (955 bp) gave 14 informative SNPs (9 substitutions and 5 indels), all of which occurred between *Glyptostrobus* and *Taxodium*. There was no variation among the *Taxodium* accessions, except for a 63 bp insertion in var. *imbricarium* (FL, *Adams 12825*)

The trnS-trnG intron (821bp) had 12 SNPs (9 substitutions and 3 indels). All 12 SNPs were between *Glyptostrobus* and *Taxodium*. No variation was found among the *Taxodium* accessions.

Sequencing of ycf exon 3 - spacer - psbA (591bp) yielded 9 SNPs (8 substitutions and 1 indel). All 9 SNPs were between *Glyptostrobus* and *Taxodium*. No variation was found among the *Taxodium* accessions, except in the long stretch of poly mono-nucleotide Ts (imb = imbricarium, dist = distichum, muc = mucronatum): 19T - imb FL 12826; 18T - dist Guad R 12848; 17T - dist 12864 AL, imb FL 12824, 12825, muc 12591 Guat., dist Sab R 12853, muc Bolleros 12838, 12839, muc Nazas 12833, 12834; 16T - dist Progresso 12843; 15T - dist AL 12868, dist LA 12862, dist L. Cherokee 12858, 12861, imb AL 12873, 12874, imb FL 12823; and 14T - dist AL 12872. There seems no regional or taxonomic pattern in the length of the poly T section. It should be noted that poly mono-nucleotides of greater than 8-10 nucleotides are difficult to sequence due to slippage of the *Taq* enzyme. But in each instance, the poly T region was sequenced in both directions and the number of Ts verified in both sequences. This may just be a hyper-variable region with no taxonomic significance.

Tsumura et al. (1999) used cleaved amplified polymorphic sequences (CAPS) to examine var. *distichum* and var. *imbricarium* in Florida (one population, Fargo, GA, was on the Georgia / Florida

border). They were able to classify each population of the two varieties except the Fargo, GA population that clustered by itself. They concluded that CAPS did support the recognition of varieties (var. *distichum* and var. *imbricarium*), but not distinct species.

Kusumi et al. (2010) examined populational variation along the Mississippi River in *T. distichum,* plus one population of *T. d.* var. *imbricarium* near New Orleans. They did not comment on the taxonomic implications of their data.

Lickey and Walker (2002) used allozymes to examine var. *distichum* and var. *imbricarium* and showed var. *imbricarium* to be somewhat distinct from var. *distichum* (clustering with var. *distichum* from Stone Mt., MS). A population of *T. mucronatum* (Sonora, MX) clustered well within *T. distichum*. Interestingly, they found that var. *distichum* from the Guadalupe River, TX had a unique allozyme pattern in their data set. They concluded that gene flow is occurring between the two varieties and that the taxa are likely varieties not species.

In our previous study on leaf terpenoids, we found (Fig. 3) considerable differentiation in the Guatemala - Oaxaca, and Durango populations from the central group of *T. distichum* in the USA. The var. *imbricarium* from Florida was only slightly different from var. *distichum* in the south-central USA (Fig. 3, 0.90) as was the Hill country - RGV populations (Fig. 3, 0.86). The high similarity of the Bolleros (DF, MX) to south-central USA (Fig. 3, 0.88) may be due to the movement of germplasm by people as the trees are widely cultivated in Mexico. However, it is clear that there has been evolutionary differentiation in *Taxodium* of southern Mexico - Guatemala and Durango in the leaf terpenoids.

In view of the lack of variation in the DNA sequences (4 gene regions, 3547 bp) found in this study, there is no support for the recognition of *T. ascendens* (*T. d.* var. *imbricarium*) and *T. mucronatum* as distinct species. In short, the present data supports the recognition of *Taxodium* as a monotypic genus with three varieties: var. *distichum* (L.) Rich.; var. *imbricarium* (Nutt.) Croom; and var. *mexicanum* (Carr.) Gord. as advocated by Denny and Arnold (2007).

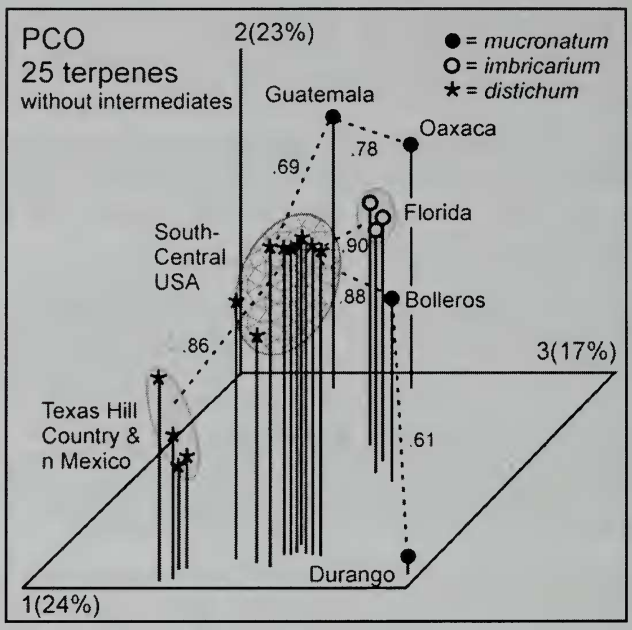

Figure 3. PCO based on 25 terpenes without intermediate terpene patterns. Adapted from Adams et al. (2012).

Key to the varieties of *Taxodium distichum* (from Denny and Arnold, 2007):

1a. Determinate short shoots mostly ascending in a vertical plane; awl-like leaves narrowly lanceolate, 2.5 - 10 mm long, appressed, and imbricate in 5 to 8 ranks on shoots.....var. *imbricarium*

1b. Determinate short shoots mostly spreading in a horizontal plane; flattened leaves narrowly linear, 5 - 15 mm long, divergent, and appearing two-ranked on shoots.....2

2a. Leaves deciduous; branches (catkins) containing male cones, short and crowded, often divided into compact secondary branches..... .....var. *distichum*

2b. Leaves semi-evergreen; branches (catkins) containing male cones, long and slender, open, made up of single cones or clusters of several cones.....var. *mexicanum*

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